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Effects of a Single Hit from the Alpha Hemolysin Produced by *Escherichia coli* on the Morphology of Sheep Erythrocytes†

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Scanning electron micrographs of sheep erythrocytes showed that attachment of the alpha hemolysin produced by *Escherichia coli* resulted in the formation of spherocytes, with 10 to 20 small projections spaced relatively evenly over the surface of the erythrocyte membrane. This shape change was induced within 5 min after treatment. If the hemolysin concentration was reduced to a level which would lyse only a fraction of the total erythrocytes, the affected cells were easily identified against a background of normal, unaffected cells. Unlike sodium lauryl sulfate and other amphipathic agents which enter cell membranes and increase their flexibility, low concentrations of hemolysin did not provide protection against hypotonic hemolysis. These findings indicate that the surface projections were not the result of membrane expansion caused by incorporation of hemolysin into the outer portion of the lipid bilayer. The ability of a given amount of hemolysin to release a constant amount of hemoglobin in the presence of increasing concentrations of red cells confirmed that a single hit is sufficient for lysis. These results suggest that a single hemolysin molecule can bind to a sheep erythrocyte and trigger internal reactions which result in the derangement of membrane integrity at multiple sites on the surface. Confirmation of one-hit kinetics indicates that measurement of *E. coli* hemolysin activity should be carried out at low ratios of hemolysin to erythrocyte to decrease the possibility of multiple hits on a single cell.

The predominance of hemolytic *Escherichia coli* associated with various disease processes suggests that the production of hemolysin may have a role in facilitating the invasion of host tissues or in permitting hemolytic *E. coli* to proliferate at the expense of other microbes. In our laboratory we have studied the synthesis and the mechanism of action of *E. coli* hemolysins to gain information as to the role of hemolysin in diseases caused by or associated with hemolytic *E. coli*. In previous studies we have shown that the cell-free (alpha) hemolysin produced by *Escherichia coli* 4331, a strain associated with edema disease of swine (12), is synthesized by bacterial modification of a high-molecular-weight component of meat broth medium rather than by synthesis de novo within the bacterial cell (11). The hemolysin has several physical and chemical properties in common with its precursor, but little is known about the mechanisms by which precursor is converted to hemolysin or by which the active hemolysin lyses erythrocytes.

The objective of this study was to examine the interaction between *E. coli* hemolysin and erythrocytes to learn more about the mechanism of action and thereby gain further insight into the nature and specific function of the hemolysin molecule. Others (14, 19) have shown that the cell-free hemolysin binds to target cells and is used up in the lytic process. In the work reported here, we used a scanning electron microscope to observe the effects of alpha hemolysin on the external surface of sheep erythrocyte membranes. Our micrographs showed that, within 5 min after treatment with hemolysin, each erythrocyte develops 10 to 20 small projections on the outer surface of its membrane. These observations prompted further studies to determine how these projections were generated. Three alternative mechanisms were considered: (i) the projections were the result of membrane expansion induced by the incorporation of large amounts of hemolysin into the outer surface of the erythrocyte membrane; (ii) each projection was the result of a hit by hemolysin at that site on the membrane surface; or (iii) a single hit by one hemolysin could cause the formation of multiple projections on the cell surface. The experiments described here indicate that the third alternative is the mechanism by which *E. coli* hemolysin induces morphological changes in sheep erythrocytes.

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MATERIALS AND METHODS

Strains and media. A strain of *E. coli*, serotype O141:85ac:NM (Minnesota stock culture no. 4331) was used. It was originally isolated from the intestine of a pig with edema disease (12) and has been maintained on agar slants. Bacteria were grown on meat extract broth prepared as previously described (11).

Hemolysin preparation. *E. coli* strain 4331 was added to meat broth cultures to give a final concentration of 10^6 cells per ml, and the growth flasks were incubated for 5.5 h at 37°C in a water bath shaking at 120 cycles per min. All remaining steps were carried out at 4°C. After the growth period, a total of 200 ml of culture medium was centrifuged at 15,000 × *g* for 15 min. The supernatant fluid was filtered through a sterile membrane with 0.45-μm pores (Millipore Corp., Bedford, Mass.). Ammonium sulfate was added to 50% saturation, and the precipitated protein was removed by centrifugation. The protein pellet was suspended in 20 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, pH 7.5, containing 0.11 M sodium chloride (TBS). The redissolved precipitate was applied to a column of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) with packed gel dimensions of 12.5 cm^2 by 50 cm. Fractions were eluted with TBS at a flow rate of approximately 12 ml/h. Eluant fractions containing hemolysin activity were pooled and lyophilyzed before storage at −5°C.

Assay of hemolysin activity. Hemolysin was measured on the basis of the amount of hemoglobin released in a solution containing 1% sheep erythrocytes as described by Short and Kurtz (19). Erythrocyte concentrations were checked and adjusted by measurement of the A_{540} of a 0.1-ml portion which had been diluted and lysed with 0.1 M NH_4OH. Blood with a hematocrit of 32 and an absorbance at 540 nm equivalent to 130 was used as a point of reference. An extinction coefficient of 14.7×10^3 M^{-1} cm^{-1} was used for calculation of the concentration of hemoglobin monomer. Estimates of the numbers of cells present in the assay mixture were made by calculations based on a mean corpuscular hemoglobin concentration of 10 pg (15) and by direct counting in a hemocytometer. These two methods gave average values of 4.5×10^8 and 3.4×10^8 cells per ml, respectively, in the solutions containing 1% sheep erythrocytes. Hemolysin preparations were diluted to concentrations which caused lysis of 5 to 30% of the total erythrocyte population. The Poisson distribution used to calculate the proportion of erythrocytes receiving *r* active hemolysin molecules was $P(r) = (z^r/r!)\ e^{-z}$, where *z* is the average number of active hemolysins per erythrocyte (9).

Preparation and fixation of erythrocytes for scanning electron microscopy. A heparinized syringe was used to draw blood from adult Suffolk sheep. The cells were washed two times with Eagle solution and a third time with TBS containing 0.02 M calcium chloride. After the final wash, the cells were suspended in the same Tris buffer at a concentration of 2% erythrocytes. A 1-ml amount of this suspension was added to 1 ml of hemolysin, which was also diluted in TBS containing 0.02 M calcium chloride. Two kinds of control incubations were run, first using buffer without hemolysin and second using buffer with hemolysin but without calcium. Both the treated and the control erythrocyte mixtures were divided, and one portion was allowed to continue incubating for the regular 1-h assay period so that the final percent lysis could be calculated. In the remaining portion, the reaction was stopped after either 5 or 10 min by the addition of 0.6 ml of the mixture to 7 ml of 1% glutaraldehyde in Eagle solution. Fixation was carried out at room temperature for 30 min, and then washing, postfixation, and dehydration steps were continued as described by Bessis and Weed (4). The dehydrated cells were suspended in propylene oxide, and a small drop of the suspension was placed on a cover slip attached to a stub. The preparations were placed in a Denton vacuum DV-502 chamber and coated by vacuum evaporation of carbon followed by gold palladium. The samples were examined with a scanning electron microscope (JEOL JSM-35, Medford, Mass.) at 15 or 25 kV.

RESULTS

Scanning electron micrographs of sheep erythrocytes after treatment with hemolysin. Sheep erythrocytes from the control cultures incubated for 5 min without hemolysin show that the cells appear as normal biconcave disks with smooth surfaces (Fig. 1A). Cells from the controls which contained hemolysin but lacked calcium also appeared normal. As noted by others working with sheep erythrocytes (10), a few (<0.5%) of the normal cells had a single small protuberance on the surface such as is shown by the white spot on the cell in the upper right of Fig. 1A. Scanning electron micrographs taken of samples fixed 5 min after the addition of *E. coli* alpha-hemolysin (0.5 U/ml) showed that each cell had 10 to 20 small projections, approximately 0.15 μm in diameter, spaced relatively evenly over the membrane surface (Fig. 1B-D). Coincident with the formation of these surface projections, the cells became spherical. Although the photographs in Fig. 1 were all taken of samples fixed 5 min after the addition of hemolysin, the treated cells have shapes varying from disks to spheres, and the projections range from tiny buds (Fig. 1C) to tear-shaped droplets and microspherules which have pulled away from the cell surface (Fig. 1D). In these experiments the amount of hemolysin added was more than enough to cause lysis of all of the cells in the incubation mixture so that each cell may have received multiple hits by hemolysin.

Inability of hemolysin to protect against hypotonic hemolysis. The apparent membrane expansion in the form of projections and droplets observed in Fig. 1 suggested that low concentrations of hemolysin might be incorporated into the membrane, thereby according increased flexibility and protection from hypotonic hemolysis. Such action has been demonstrated

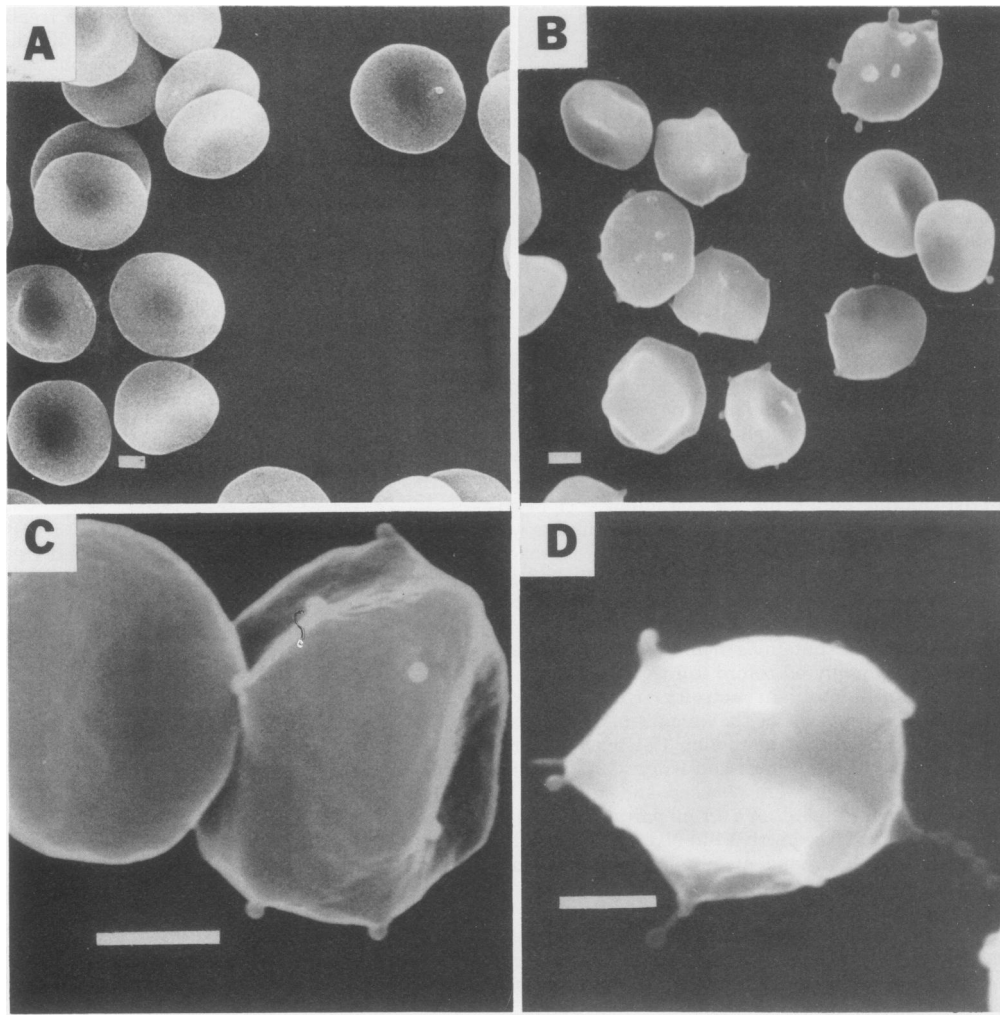

FIG. 1. *Scanning electron micrographs of sheep erythrocytes from incubation mixtures fixed after a 5 min incubation period with TBS (A) or with E. coli alpha hemolysin diluted in TBS (B, C, D). Bar, 1 μm.*

for several nonspecific lipid soluble lysins (16) and was reproduced in our laboratory by using sodium lauryl sulfate. These assays were run by the procedure described by Seeman, except that sheep erythrocytes, suspended in 0.01 M Tris-chloride buffer, pH 7.5, with 0.154 M NaCl and 0.02 M calcium, were used in place of human cells. Portions (0.2 ml each) of this 20% suspension were added to 3 ml of the test solution containing 0.01 M Tris-chloride, pH 7.5, 0.05 M NaCl, 0.02 M calcium chloride, and various concentrations of sodium lauryl sulfate (Sigma Chemical Co., St. Louis, Mo.), saponin (Matheson, Coleman and Bell, Norwood, Ohio), or hemolysin. A concentration of 10^{-4} M sodium lauryl sulfate caused a marked increase in the resistance of the erythrocytes to hypotonic hemolysis (Fig. 2). In these experiments, the relative hemolysis plotted on the ordinate was obtained by dividing the amount of hemoglobin released in the presence of the lysin by the amount of hemoglobin released in the control. In contrast to sodium lauryl sulfate, the effects of *E. coli* hemolysin were more like those of saponin, a specific hemolysin which does not become incorporated into the plasma membrane. Neither hemolysin nor saponin provided any evidence of protection from hypotonic he molysis.

Evidence for lysis of an erythrocyte after a single hit by hemolysin. Previous data suggest that a single hemolysin molecule is suffi cient for lysis of a sheep erythrocyte and that the hemolysin molecule is used up in the lytic

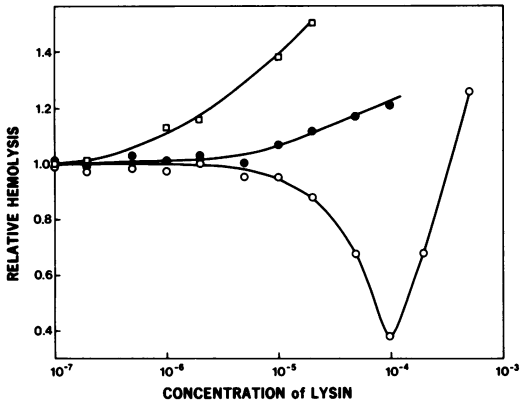

FIG. 2. *Ability of low concentrations of lytic agents to stabilize erythrocytes against hypotonic hemolysis. The numbers on the abscissa correspond to molarities of sodium lauryl sulfate* (○) *and saponin* (□) *and to dilutions of a hemolysin preparation with an original activity of 100 units per ml* (●).

process (19). This hypothesis was confirmed by an experiment patterned after the procedure used by Inoue et al. (9). A commercial preparation of sheep erythrocytes (Wilfer Laboratories, Stillwater, Minn.) was washed three times with TBS containing 0.02 M calcium, and the cells were suspended in the same buffer to give a series of final concentrations of 16, 8, 4, 2, 1, 0.2, and 0.1% erythrocytes. Hemolysin was also diluted in TBS containing 0.02 M calcium. Each incubation vessel contained 1 ml of erythrocyte suspension and 1 ml of diluted hemolysin. Blanks which contained buffer without hemolysin were run for each concentration of erythrocytes. All tubes were incubated at 37°C for 1 h. The cells were removed by centrifugation at 1,000 × *g* for 10 min, and the absorbance at 540 nm of the supernatant fluid was recorded. The two curves in Fig. 3 show the total amounts of hemoglobin released by 0.1 (upper curve) and 0.05 (lower curve) U of hemolysin in the presence of increasing numbers of erythrocytes in a constant reaction volume. In both cases, the amount of hemoglobin released rises to a maximum value and then remains constant despite a doubling of the number of erythrocytes. At high cell concentrations, the absolute number of cells lysed is essentially independent of the total number of erythrocytes added which confirms that the reaction proceeds by a one-hit process. These curves also show that the addition of twice as much hemolysin causes the release of twice as much hemoglobin. Assuming that each erythrocyte releases 10 pg of hemoglobin, the maximum number of cells lysed by the 0.1 and 0.05 U of hemolysin is 17×10^7 and 8×10^7 cells, respectively.

Identification of erythrocytes hit by hemolysin. Confirmation of the ability of a single hit to cause lysis prompted reexamination by scanning electron microscopy, using a population of erythrocytes which had been treated with a limited amount of hemolysin. Under conditions in which the hemolysin is sufficient for lysis of only a limited percentage of the total number of cells present, cells which have been hit should be distinguishable from those which retain their normal morphology. Figure 4 shows one of the scanning electron micrographs of cells fixed 10 min after the addition of an amount of hemolysin sufficient for lysis of 20% of the total erythrocytes present. These micrographs showed that, whereas most of the cells retained their normal shape, approximately 20 to 30% were affected by hemolysin. Each of the affected cells has many projections and bulges and can be clearly distinguished from the remainder of the cells which appear as normal biconcave disks. From the Poisson formula (9), it can be calculated that under conditions in which 20% of the cells are lysed, less than 5% will be hit by more than one molecule of hemolysin. Thus the multiple projections on a single cell are not the result of multiple hits.

DISCUSSION

The data in Fig. 3 which confirm that lysis is caused by a single hit also indicate that the morphological changes observed in the prelytic phase (Fig. 1 and 4) are caused by attachment of a single molecule of active *E. coli* alpha-hemolysin. In this prelytic period, the development of multiple projections spaced relatively evenly

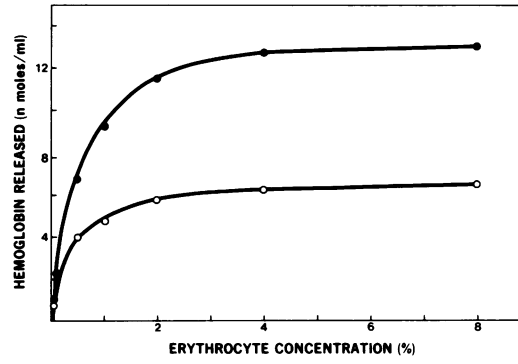

FIG. 3. *Release of hemoglobin by constant amounts of hemolysin in the presence of increasing numbers of erythrocytes. Incubation mixtures contained erythrocytes at a final concentration indicated on the abscissa and either 0.1* (●) *or 0.05* (○) *U of hemolysin in a final volume of 2 ml. Each point represents the average reading from 3 tubes minus the average of 3 blank tubes without hemolysin.*

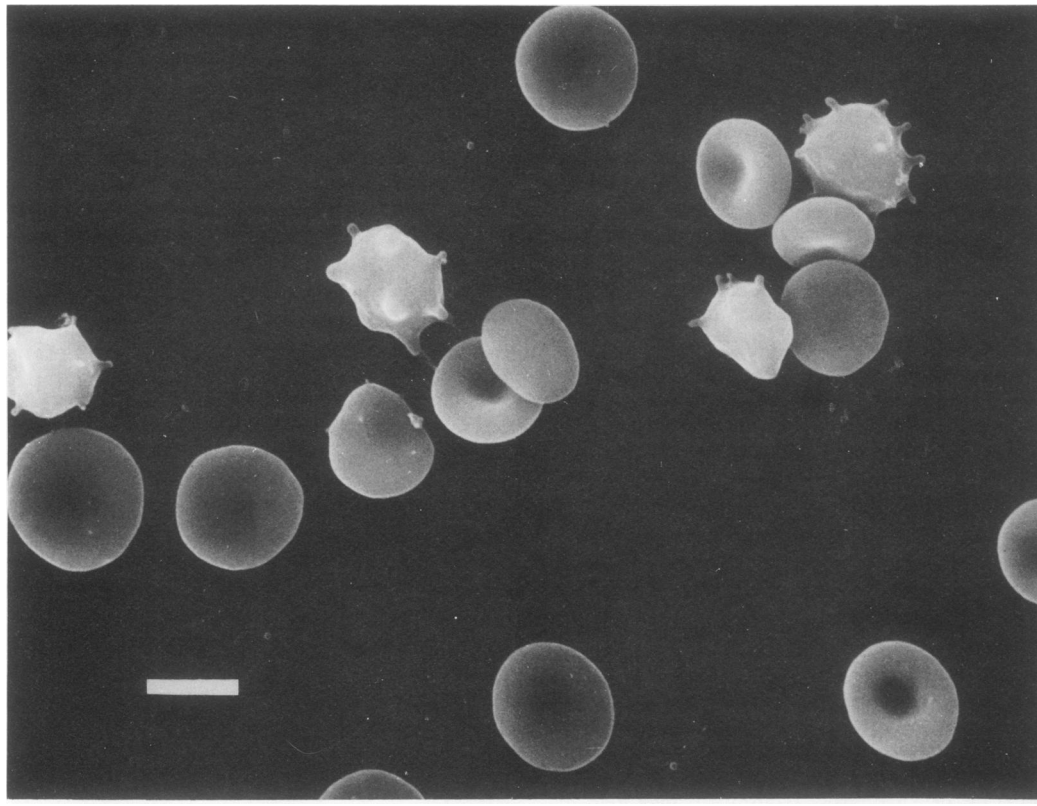

FIG. 4. *Scanning electron micrograph of sheep erythrocytes from an incubation mixture fixed 10 min after the addition of hemolysin. The ratio of cells to active hemolysin moieties was approximately 5 to 1. Bar, 3 μm.*

over the outer membrane surface and the concomitant shape change from a disk to a sphere are similar to the formation of echinocytes which has been described extensively in studies of human erythrocytes (3) and has been shown to be caused by a variety of mechanisms (1, 13, 18, 22, 23). One of these mechanisms is by expansion of the cell membrane by incorporation of external molecules into the outer surface of the lipid bilayer (17, 22). Our data indicate that *E. coli* hemolysin does not act in this manner, since a single molecule was effective in inducing the formation of multiple surface projections. In addition, low concentrations of hemolysin did not afford any protection against hypotonic hemolysis such as is characteristic of other echinocytic agents which enter and expand the outer portion of the membrane surface (Fig. 2) (16).

In our system, the efficacy of a single hit and the fact that major morphological changes occur within 5 min suggest that hemolysin mediates its action by altering internal reactions within the sheep erythrocyte. The action of hemolysin as visualized in the scanning electron micrographs are consistent with an effect on either the spectrin-actin cytoskeleton or on the lipid components of the membrane (2). There are at least two mechanisms whereby hemolysin might mediate either of these kinds of changes in the internal metabolism of the erythrocyte. The hemolysin could act as an ionophore which facilitates a sudden change in ion concentration across the plasma membrane. The high concentrations of calcium required for hemolysin activity (0.02 M) (19) and the low calcium levels within normal sheep erythrocytes (3×10^{-5} M) (7) are consistent with a mechanism whereby attachment of hemolysin creates a channel, allowing a sudden influx of calcium into the cell. However, previous work of Eaton et al. (7) has shown that, unlike other mammalian erythrocytes, a sudden influx of calcium ion does not cause a shape change in sheep erythrocytes. Thus, if ion transport occurs, something other than calcium may be involved. Such an alteration in ion concentration might then either activate a lipase, thereby disrupting membrane lipids, or stimulate an adenosine triphosphatase,

causing a rapid depletion of adenosine triphosphate and thus effecting a change in the conformation of the spectrin-actin network. Alternatively, hemolysin may have subunits such that a binding component remains on the surface and an active portion enters the cell and alters a specific enzymatic activity in a manner similar to that described for several other toxins (6, 8). Postulation of separate binding and active subunits would account for the fact that the hemolysin molecule seems too large to enter the cell intact (molecular weight = 120,000) (11, 24) and the fact that each hemolysin is inactivated in the lytic process (19).

A better understanding of the way in which *E. coli* hemolysin interacts with erythrocytes might provide information about the molecular structure of hemolysin, which in turn could aid in assessing its role in the pathogenesis of edema disease. Knowledge of the specific mechanism of action of hemolysin may also make it a useful tool for the study of membrane structure.

The findings described in this paper suggest an important factor to be considered when designing assays for quantitation of hemolysins which bind to the cell surface and work by a single-hit mechanism such as the hemolysin produced by *E. coli*. At very low concentrations of erythrocytes, when the relative ratio of hemolysin to cells is high, the amount of hemoglobin released does not give a valid indication of the amount of hemolysin present (Fig. 3). Under these conditions where virtually all of the cells are lysed, most of the hemolysin is absorbed onto cells which have already been hit and is thus not detected. The values given in Table 1 have been calculated by using the Poisson formula to show the relative amounts of hemolysin that would be masked in assay vessels where the ratio of hemolysin to erythrocytes is increased to allow lysis of larger percentages of the total erythrocyte population. These figures indicate that traditional assays for hemolysin which are based on the highest serial dilution that gives 50 or 100% lysis would not provide an accurate means of measuring the amount of a hemolysin which works by a one hit mechanism and is used up in the lytic process. Therefore, in earlier studies of *E. coli* hemolysin (14, 20, 21, 24), the amount of activity may have been considerably underestimated. With an assay system in which the ratio of hemolysin-to-erythrocytes is relatively low, the actual number of active hemolysin molecules in the preparation can be calculated by measurement of the number of cells lysed.

TABLE 1. *Effect of hemolysin-to-erythrocyte ratios on detection of E. coli hemolysin activity*

% of total cells lysed	Hemolysin/erythrocyte (z)[a]	% of hemolysin not detected[b]
10	0.106	5
20	0.224	11
30	0.356	16
40	0.51	22
50	0.69	28
90	2.3	61
95	3.0	68
99	4.6	78

[a] Calculated from $P_{(o)} = e^{-z}$, where $P_{(o)}$ = (100 − percent lysed)10^{-2}.

[b] Each value represents 100 − (percent lysed/100z).

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